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Isolation and cDNA cloning of a novel red colour-related pigment-binding protein derived from the shell of the shrimp, *Litopenaeus vannamei*

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ABSTRACT

Pigment-binding proteins play important roles in crustacean shell colour change. In this study, a red colour-related pigment-binding protein, designated LvPBP75, was purified from the shell of *Litopenaeus vannamei*. HPLC and PAGE analysis showed that LvPBP75 was a homogeneous monomer with molecular mass of 75 kDa. Peptide mass fingerprint analysis revealed that LvPBP75 belonged to hemocyanin, and the released pigment from heated LvPBP75 showed a λ_{max} at 481 nm in acetone. The significant red-colour change temperatures were detected at 30 and 80 °C, respectively. Based on the determined amino acid fragments, a full-length cDNA of LvPBP75 was cloned and sequenced. The ORF encodes a protein of 662 amino acids having 80% identity with penaeidae hemocyanin. These results strongly suggest a novel function of hemocyanin, namely binding with pigment, and its involvement in *L. vannamei* shell colour change.

1. Introduction

The colour of crustacean shells changes from dark blue to bright red-orange when cooked (Ertl et al., 2013; Wade et al., 2012). In addition to reflecting the freshness of crustaceans, the red colour change also plays a significant role in consumer acceptability of commercial crustacean species (Erickson, Bulgarelil, Resurreccion, Vendetti, & Gates, 2007; Parisenti et al., 2011). A pioneering study on lobster shell revealed that this well-known red colour change is due to the release of pigments from denatured pigment-binding proteins, named crustacyanin (Chayen et al., 2003; Cheesman et al., 1966; Durbeej & Eriksson, 2003; Elizabeth, David, & Andrew, 2009; Wade, Tollenaere, Hall, & Degnan, 2009; Wald & Nathanson, 1948; Zagalsky, 1985). Crustacyanin extracted from a lobster shell comprises carotenoproteins and pigments (Wald & Nathanson, 1948). The predominant carotenoprotein in the lobster shell is α-crustacyanin (λ_{max}: 632 nm, ~320 kDa), which contains 16 protein subunits and 16 astaxanthin molecules (Chayen et al., 2003; Zagalsky, 1985). After irreversible dissociation, β-crustacyanin (λ_{max}: 585 nm, ~40 kDa) appears; eight units of β-crustacyanin can form one unit of α-crustacyanin. Both α- and β-crustacyanin can easily dissociate into apoproteins (~20 kDa) under experimental conditions (Elizabeth et al., 2009).

Crustacean shell colour change was recently shown to be determined by crustacyanin and the interaction between pigments and proteins (Michael, Anita, Sara, Sutara, & Saskia, 2009; Parisenti et al., 2011; Reszczynskaab, Welca, Grudzinskia, Trebaczb, & Gruszeckia, 2015; Wade, Melville-Smith, Degnan, & Hall, 2008). In nature, pigment-binding proteins can be influenced by the amount of carotenoids in the daily diet, background substrate colour, light intensity, water quality and growth stage (Chayen et al., 2003; Palma & Steneck, 2001; Wade et al., 2008, 2009, 2012). It was reported that American lobster (*Homarus americanus*) shell colour is mainly influenced by ultraviolet light; moreover, in the absence of ultraviolet light, the lobster shell matches the background colour after long-term exposure (Michael et al., 2009). Meanwhile, the mechanism of shell colour change in prawn (*Penaeus monodon*) has been reported to be strongly correlated to the background substrate colour (Wade et al., 2012). In addition, a study on the different body colours of burrowing crab (*Neohelice granulata*) revealed that the colours are determined by its habitat (Casariego, Luppi, & Iribarne, 2011). Furthermore, crustacean shell colours are associated with body size, molting stage, and migration (Yanar, Çelik, & Yanar, 2004; Zadorozhny, Borisovets, Yakush, & Davidyuk, 2008). When crustaceans are cooked, their shell colours are influenced by storage conditions, heating temperatures, processing methods and

Abbreviations: DTT, dithiothreitol; IAA, iodoacetamide; MALDI-TOF-MS, matrix assisted laser desorption ionization/time of flight tandem mass spectrometry; PMF, peptide mass fingerprinting; TFA, trifluoroacetic acid

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food additives (Ando, Fukai, Kawasaki, Itoh, & Tsukamasa, 2014; Brookmire, Mallikarjunan, Jahncke, & Grisso, 2013; Huang, Mirtala, & Shiau, 2016). Even though crustacean shell colour change is associated with the release of pigment, pigment-binding proteins and protein-pigment interactions differ among crustacean species (Begum et al., 2015; Nur-E-Borhan, Okada, Watabe, & Yamaguchi, 1995; Porter, Bok, Robinson, & Cronin, 2009; Velu, Czeczuga, & Munuswamy, 2003).

Pacific white shrimp (*Litopenaeus vannamei*) is one of the economically important species of shrimp culture. It is mainly cultured in Asian countries, both as a food item for domestic consumption and as a valuable export commodity (Nirmal & Benjakul, 2009). Although it is well known that *L. vannamei* shell colour changes from pale gray to bright red-orange after cooking (Sriket, Benjakul, Visessanguan, & Kijroongrojana, 2007), the underlying mechanism and red colour-related pigment-binding proteins derived from *L. vannamei* shell are poorly understood. Accordingly, in the present study, we isolated and purified a novel red colour-related pigment-binding protein from the shell of *L. vannamei* by ammonium sulfate precipitation, gel filtration and anion exchange HPLC. Then, we determined the red colour change ability of this pigment-binding protein by subjecting it to different heat treatments. Furthermore, we clarified the primary structure of the binding protein using cDNA cloning.

2. Materials and methods

2.1. Materials

Eighty Pacific white shrimp (*L. vannamei*) that were 2.0 g in body weight, and 6 cm in body length were collected in living status from a farm in Myoko City, Niigata prefecture, Japan. They were transported to the lab and acclimated in tanks containing aerated artificial seawater (2.5%) for 24 h before tissue collection.

2.2. Extraction of proteins derived from L. vannamei shell

2.2.1. Water-soluble proteins

Water-soluble proteins were prepared as described by Zagalsky (1985) and Elizabeth et al. (2009) with some modifications. Briefly, specimens of shrimp *L. vannamei* were purchased from the Tokyo Central Wholesale Fish Market (Tokyo, Japan). Shells were obtained, thoroughly freed from the underlying tissue by scrubbing with ice water, and dried overnight at 4 °C. The shells were minced with an electric food processor, and soaked for 16 h with agitation in 0.3 M boric acid adjusted to pH 6.8 with solid Tris and then transferred into pre-cooled (4 °C) 10% (w/v) EDTA solution (pH 7.0; 25 g shell with 1000 ml solution). The shells were removed by filtration after overnight stirring and the filtrate was brought to 60% saturation with ammonium sulfate. After stirring overnight at 4 °C, the precipitate was collected by centrifugation at 15,000 × *g* for 20 min at 4 °C, and then re-suspended in 50 mM phosphate buffer (pH 7.0). The interfering proteins were removed by precipitation with ammonium sulfate to 30% saturation. The blue-coloured filtrate, containing water-soluble proteins, was precipitated by increasing the saturation to 60%.

2.2.2. Non-water-soluble proteins

In addition to the traditional water-soluble proteins, non-water-soluble proteins were also scanned for the existence of red colour-related proteins. They were divided into salt-, acid-, and alkaline-soluble parts based on protein solubility and extracted according to Fernlund's method (Fernlund & Josefsson, 1968). 30% interfering precipitate was re-dissolved into 50 mM phosphate buffer containing 1 M NaCl and the salt-soluble extract was collected by centrifugation at 15,000 × *g* for 20 min at 4 °C. The precipitate was soaked in 0.1 M HCl or NaOH to extract acidic- and alkaline-soluble proteins, respectively. All precipitate obtained from the previous step was washed with distilled water (pH 7.0) three times before being subjected to subsequent extraction experiments.

2.3. Purification of red colour-related protein

The crude water-soluble proteins were subjected to gel filtration HPLC on a Superdex 200 10/300 GL column (1 × 30 cm, GE Healthcare Biosciences, Buckinghamshire, UK) that was equilibrated and washed with 0.15 M NaCl/50 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. Red colour-related fractions were collected and subjected to HPLC on a Mono Q 5/50 GL column (5 × 50 mm, GE Healthcare Biosciences); the column was equilibrated with 50 mM phosphate buffer (pH 7.0) and developed by a linear gradient of NaCl (0–1 M over 50 min) in 50 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. At each chromatographic step, the proteins were monitored by recording A_{280} and the red colour change was determined after heating in a 100 °C hot water bath by using a colorimeter (CLR-7100F, Shimadzu, Kyoto, Japan). The purified red colour-related protein was termed as LvPBP75.

LvPBP75 was analyzed for its homogeneity by PAGE. Native-PAGE was performed on a 12% handmade gel. SDS–PAGE was carried out on a 5–20% precast gel (Atto, Tokyo, Japan) with an AE-7300 (Atto, Japan) compact PAGE system. Before SDS–PAGE, LvPBP75 was mixed with an equal volume of 0.125 M Tris–HCl buffer (pH 6.8) containing 4% SDS and 10% 2-mercaptoethanol and heated in a boiling water bath for 5 min. After being run, the gel was stained with a Rapid Coomassie Brilliant Blue R-250 (Kanto Chemical Co., Inc. Tokyo, Japan), and Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as a reference.

The molecular mass of non-denatured LvPBP75 was determined by gel filtration HPLC using a TSKgel G3000 SW_{XL} column (0.78 × 30 cm, Tosoh, Tokyo, Japan) with 0.15 M NaCl/50 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min and monitored by recording A_{280}. Five reference proteins were used to calibrate the column: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *C* (12.4 kDa) (Sigma-Aldrich, St. Louis, MO, USA). The molecular mass of denatured LvPBP75 was determined by SDS–PAGE as described above.

2.4. Colour shift of the proteins derived from shrimp shell

Investigation of the colour shift of the proteins derived from shrimp shell was performed by heating at temperatures of 30, 45, 60, 80, and 100 °C for 10 min. Unheated samples were set as controls. Temperatures and colour changes were monitored and recorded. Colour changes were investigated by using the colorimeter (CLR-7100F). The results are expressed as L^* (brightness), a^* ($+a$ red, $-a$ green), and b^* ($+b$ yellow, $-b$ blue).

The pigments released from cooked LvPBP75 were extracted based on the method of Yanar et al. (2004) with some modifications. After heating, the red coloured precipitate was collected by centrifugation at 10,000 × *g* for 10 min at room temperature. Then it was mixed with equal amounts of anhydrous sodium sulfate and soaked into 5 ml acetone overnight. The mixture was homogenized and centrifuged at 10,000 × *g* for 10 min. The UV–visible absorption spectra of uncooked LvPBP75 and acetone extract were determined by a micro-volume spectrophotometer from 220 to 800 nm (BioSpec-nano, Shimadzu, Kyoto, Japan).

2.5. In-gel digestion and peptide mass fingerprinting (PMF) analysis

PMF analysis of LvPBP75 was performed according to the method of Li et al. (2012) with slight modifications. The LvPBP75 spot was manually cut from the SDS–PAGE gel that was stained with CBB solution and then incubated in 10 mM DTT and 55 mM IAA in the dark for 30 min. Next, the gel pieces were de-colourized in solution A (50 mM CH_3OH:NH_4HCO_3, 1:1) then by solution B (50 mM NH_4HCO_3:Acetonitrile, 1:1) for 15 min. The gel pieces were then dehydrated with 100% acetonitrile and subsequently rehydrated with 20 ng/μl trypsin for 30 min on ice. 50 mM